https://doi.org/10.17221/201/2016-CJGPB

Variation in Ploidy Level and Morphological Traits in the Progeny of the Triploid Apple Variety Jonagold

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Abstract

He P., Li L., Cheng L., Wang H., Chang Y. (2018): Variation in ploidy level and morphological traits in the progeny of the triploid apple variety Jonagold. Czech J. Genet. Plant Breed., 54: 135–142.

Variation with respect to both ploidy level and morphology was characterized for a set of 690 seedlings of the triploid apple variety Jonagold, of which 481 were obtained via *in vitro* culture of mature embryos, and 209 via conventional germination. Their ploidy level was determined by a combination of flow cytometry and root tip chromosome counting. The assessed morphological traits were leaf length, width and shape, stomatal density, guard cell length and chloroplast width and number. A total of 452 seedlings were aneuploid, 225 diploid, nine triploid and four tetraploid. All four tetraploid seedlings were derived by *in vitro* culture. When the triploid seedlings were genotyped at the *S*-locus and at selected microsatellite loci, we found that the Jonagold stigma was compatible with pollen which shared some of the maternal parent *S*-locus alleles.

Keywords: flow cytometry; karyotypic analysis; S-alleles; SSR markers

The majority of commercial apple (*Malus domestica* Borkh.) varieties are diploid ($2n = 2x = 34$), but triploid ($2n = 3x = 51$) forms are also known (Lespinasse *et al*. 1976); the latter types tend to be more vigorous and to form larger fruits. Triploid varieties are largely self-sterile, but typically produce a small number of viable gametes carrying either $n = 17$, 34 or 51. Polyploid variants of many plant species typically differ from the diploid ones at the level of morphology, so that morphology can be used to predict the ploidy level: for example, leaf shape, stomatal size and the number of chloroplasts harboured by guard cells have all been used as predictors of ploidy level in alfalfa (Bingham 1968), clover (Najcevska & Speckmann 1968) and sugar beet (Moehizuki & Sueoka 1955). The most reliable method for establishing the ploidy level is, however, to perform a mitotic chromosome count in either the root tip or the shoot tip, although this is a rather labour-intensive and time-consuming procedure (Michaelson *et al*. 1991). An attractive alternative is to exploit the capacity of flow cytometry to quantify nuclear DNA content in non-dividing cells (Riccardi & Nicoletti 2006).

As many as 50% of angiosperm species are self-incompatible. *Rosaceae* species achieve this property via gametophytic self-incompatibility (GSI), in which the pollen phenotype is determined by its haploid genotype. The genetic basis of GSI in apple is based on the allelic constitution of the *S*-locus, which harbours at least two genes, one encoding an S-RNase (SLF) and the other an F-box protein (SFB). These genes control pistil and pollen specificity, respectively (Minamikawa *et al*. 2014).

Here, the ploidy level of seedlings of the triploid apple cultivar Jonagold was determined. Some of the seeds, derived by open-pollination, were germinated conventionally, while others were used to provide mature embryos as the explant for *in vitro* culture. The seedlings were subsequently characterized with respect to a panel of morphological traits, namely leaf length and shape, stomatal density, guard cell length and chloroplast width and number. A small number of triploid seedlings was genotyped with respect to the *S*-locus and a set of genome-wide microsatellite (SSR; simple sequence repeat) loci. The objectives were to (1) determine whether the *in vitro* culture of

https://doi.org/10.17221/201/2016-CJGPB

mature embryos represented an effective means of multiplying germplasm and obtaining novel germplasm, (2) identify morphological traits associated with ploidy level to provide a simple method for ploidy selection, (3) evaluate the variation (if any) in the *S*-locus genotype between the maternal donor plant and its triploid progeny and (4) provide polymorphisms for the Jonagold seedlings from *in vitro* mature embryo culture which had the same ploidy level and *S*-alleles as its maternal material.

MATERIAL AND METHODS

Plant material. Open-pollinated fruits were harvested from a Jonagold tree grown in an orchard where most of the trees were Jonagold. In all, 2000 seeds were obtained; the embryos were aseptically removed from 843 plump and 157 shrivelled seeds, and subjected to *in vitro* culture, while the remainder (809 plump and 191 shrivelled seeds) was subsequently after-ripening stratified and sown.

***In vitro* culture of mature embryos**. The seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 30 s, then in 0.1% (w/v) aqueous mercuric chloride for 10 min, and were finally rinsed four times in sterile distilled water. After stripping off the testa, the embryos were excised and placed on Murashige and Skoog (1962) medium (MS medium) solidified with 6 g/l agar and supplemented with 3% (w/v) sucrose, 83 mM activated carbon, 400 mg/l lactalbumin hydrolysate, 2.2 µM 6-benzyladenine (BA), 1.2 µM α-naphthaleneacetic acid and 0.57 mM ascorbic acid. Each 100 ml bottle, containing 40 ml of the medium, housed seven embryos, and was exposed to a constant temperature of 25 ± 2°C with a 14 h photoperiod provided by white fluorescent tubes providing 50 µmol m^2/s of light. The regeneration percentage after 15 days was calculated from the number of regenerants/the number of embryo explants, while the mortality percentage was calculated after 30 days from the number of dead regenerants/the number of initial regenerants.

Multiplication, rooting and transplantation. To vegetatively multiply each regenerant, 1 cm stem sections were cut and set vertically in MS medium containing 1.1 µM 6-BA and 0.1 mg/l indole acetic acid, and transferred to a fresh medium every five weeks. When the number of emerging shoots reached 20 and the shoots exceeded 3 cm in length, the shoots were removed from the stem and placed on a solidified half-strength MS medium containing 0.1 mg/l indole-3-butyric acid to encourage root growth. Rooted plantlets were planted into trays containing a growing medium, maintained at 15–24°C under a 12 h photoperiod and watered regularly.

Seed common stratification and germination. The seeds were surface-sterilized by immersion in 10% v/v commercial bleach (4.5% NaOCl) for 5 min, then rinsed three times in sterile distilled water. The seed was then mixed with three parts of autoclaved white sand, and left to stratify in sterile boxes covered with polyethylene film at 0–15°C room. After about 12 weeks, when the radicle had emerged from > 50% of the seed, the seedlings were planted into a growing medium and maintained in a greenhouse delivering 10–24°C and a 12 h photoperiod. Percentage of germination was calculated after further 30 days, along with percentage of mortality.

Morphological traits. Fifty leaves were selected, the third or fifth leaf from the every plantlet bottom. Measurements were taken of leaf length and width, and a leaf shape index was calculated. Stomatal density of 125 per µm^2 was made statistic and transformed into the quantity of 1 mm^2, by choosing 20 pieces per shoot from each leaf. Stomatal size was estimated using an ocular measuring tiny ruler and the number of chloroplasts per guard cell was counted.

Root-tip chromosome counts. Chromosome numbers were determined from root tips prepared following a protocol modified from that described by Kondo and Lavaral (1984). Excised root tips were incubated for ca. 8 h in p-dichlorobenzene saturated water solution at 18°C, then fixed for 15 min in 3:1 95% ethanol/glacial acetic acid at room temperature. Fixed root tips were hydrolyzed in 5M HCL for 3 min at 20°C, rinsed three times in distilled water, stained in carbol fuchsin for 4 min and finally squashed under a micro cover glass. A minimum of 20 well-spread metaphase plates per root tip were sampled for chromosome counting.

Leaf tissue flow cytometry. Leaf tissue was chopped with a scalpel, then homogenized in nuclear isolation buffer [10 mM $MgSO_4 \cdot 7\ H_2O$, 50 mM KCl, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 3 mM dithiothreitol (Sigma, St. Louis, USA), 100 µm/l propidium iodide, 0.25% (v/v) Triton X-100, pH 8.0] After passing the homogenate through a 30 µm nylon mesh, the material was centrifuged (15 000 rpm, 30 s), the supernatant was discarded, and the pellet was treated with 1 µl 1 mg/ml RNase A (dH_2O) for a final concentration of 1.25 µg/ml for 15 min at 37°C. Nuclear DNA contents were measured with a

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Beckman Coulter EPICS Altra flow cytometer (www.beckmancoulter.com/). Nuclei extracted from leaves of the Golden Delicious variety were treated as the internal standard, and a minimum of 10^4 nuclei per sample was analysed. Detection followed excitation with an argon laser (488 nm). The mean fluorescence of each seedling G1 phase peak was divided by the fluorescence reading of the internal control. Each sample was run in triplicate. An estimate of the relative nuclear DNA content was derived from the ratio between the sample G1 and the internal standard G1.

PCR-based genotyping. DNA was extracted from young leaves of the triploid *in vitro* mature embryo culture-derived progenies using the method given by Koller *et al.* (2000), and diluted to 50 ng/µl. For *S*-locus (on LG17) genotyping, each 20 µl PCR comprised 1× PCR buffer (Promega, Shanghai, P.R. China), 1.75 mM $MgCl_2$, 200 µM each dNTP, 1 µM each primer (sequences given in Table 1), 0.6 U *Taq* DNA polymerase (Promega, Shanghai, China) and 100 ng template. The reactions were first denatured (94°C/3 min), then subjected to 30 cycles of 94°C/30 s, 60°C/60 s and 72°C/60 s, and finally to 72°C/10 min. The amplicons were electrophoresed through a 1.5% TBE/agarose gel and visualized by EtBr staining. For the five SSR assays (CH01b12 on LG4, LG12 or LG13, CH01e12 on LG8, CH01f02 on LG12, CH01f09 on LG8 and CH02d12 on LG11), the PCR volume was reduced to 15 µl, containing 50 ng genomic DNA, 10 mM Tris-HCl pH 9.0, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 µM each primer (Table 1) and 1 U *Taq* polymerase (Tianwei, Beijing, P.R. China). The amplification profile included an initial denaturation (94°C/3 min), followed by four cycles of 94°C/30 s, 65°C/60 s, 72°C/60 s, with the annealing temperature falling by 1°C per cycle; further 30 cycles of 94°C/30 s, 60°C/60 s, 72°C/60 s were then given, and the reaction was completed by a final extension of 72°C/5 min. The amplicons were denatured by the addition of 1 volume of denaturing gel loading buffer and holding at 94°C for 5 min; they were then electrophoresed through a 6% denaturing polyacrylamide gel. Fragments were visualized by silver staining, following Bassam *et al.* (1991).

Experimental design and data analysis. All data were subjected to the analysis of variance for a randomized complete block design prepared and analysed by the SPSS program (version 10.0, 1999). The data were expressed as the mean and standard deviation (SD).

RESULTS

Gaining seedlings from *in vitro* culture and common stratification and germination. A comparison of the recovery rate of seedlings following *in vitro* culture or common stratification and germination is shown in Table 2. For the former, 632 out of the 843 plump seeds (73.9%), but only 89 out of the 157 shrivelled seeds (56.7%) developed into a regenerant. The equivalent frequencies following common stratification and germination were 32.9% (266/809) and 17.8% (34/191). Not all of the seedlings/regenerants were viable: the mortality rate of plants recovered from shrivelled seeds was higher than that of plants recovered from plump seeds. In all, 690 viable plants were obtained, of which 481 were the outcomes of *in vitro* culture and 209 of common stratification and germination, suggesting a twofold efficiency advantage of the tissue culture approach.

Variation in mitotic chromosome number in the Jonagold progeny. The euploid seedlings carried either $2n = 2x = 34$, $2n = 3x = 51$ or $2n = 4x = 68$, while a large number of aneuploids was also detected (Figure 1, Table 3). Of the 690 seedlings, four (0.6%)

Table 1. Sequences of PCR primers in Jonagold

SSR locus name	Primer sequence 5'→3'	*S*-allele name	Primer name	Primer sequence 5'→3'
CH01B12	cgcatgctgacatgttgaat cggtgagccctcttatgtga	*S1*	FTC168 FTC169	atattgtaaggcaccgccatatcat ggttctgtattggggaagacgcacaa
CH01E12	aaactgaagccatgagggc ttccaattcacatgaggct	*S2*	OWB122 OWB123	gttcaaacgtgacttatgcg ggtttggttccttaccatgg
CH01F02	accacattagagcagttgagg ctggtttgttttcctccagc	*S3*	FTC177 FTC226	caaacgataacaaatcttac tatatggaaatcaccattcg
CH01F09	atgtacatcaaagtgtggattg ggcgctttccaacacatc	*S5*	FTC10 FTC11	caaacatggcacctgtgggtctcc taataatggatatcattggtagg
CH02D12	aaccagatttgcttgccatc ctggtggtaaacgtggtg	*S9*	FTC154 FTC155	cagccggctgtctgccactt cggttcgatcgagtacgttg

https://doi.org/10.17221/201/2016-CJGPB

Table 2. The production and mortality of apple regenerants/seedlings following either *in vitro* culture or conventional germination

Culture method	Seed mature level	No. of cultured seed	No. of plantlet	Plantlet rate (%)	No. of death plantlet	Death rate (%)
In vitro culture	plump-seeds	843	623	73.9	187	30
	shriveled-seeds	157	89	56.7	44	49.4
Common stratification and germination	plump-seeds	809	266	32.9	74	27.8
	shriveled-seeds	191	34	17.8	17	50

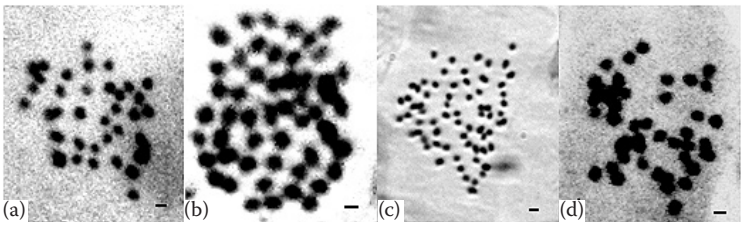

Figure 1. Variation of chromosome number in different ploidy levels of Jonagold seedlings: $2n = 34$ (a), $2n = 51$ (b), $2n = 68$ (c), $2n = 40$ (d); bar: 10 μm

were tetraploid, nine (1.3%) triploid, 225 (32.6%) diploid and 452 (65.5%) aneuploid. Although euploid individuals were recovered following both *in vitro* culture and conventional germination, the representation of diploid individuals was fivefold greater in the former than in the latter progeny set. All four tetraploids recovered emerged from *in vitro* culture.

Variation in nuclear DNA content among the Jonagold progeny. The relative nuclear DNA content of young leaf cells was tested using flow cytometry for a set of 100 karyotyped seedlings: of these, 13 were tetraploid/triploid, 30 were diploid and 57 were aneuploid. The test was based on an internal standard provided by the G1 phase peak value of an established ploidy species within the cultivar Golden Delicious, which is diploid and the nuclear DNA content of Golden Delicious was 47.2. Four representative outcomes are illustrated in Figure 2: here,

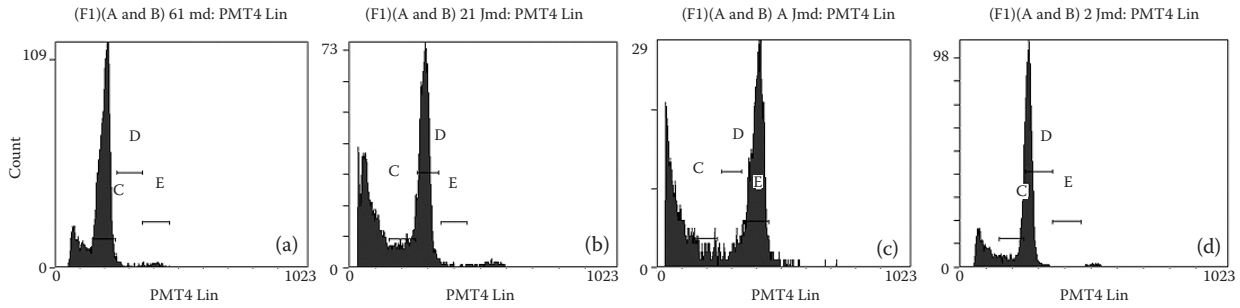

Figure 2. Relative nuclear DNA contents inferred from the flow cytometry-based analysis of nuclei isolated from young leaves of Jonagold seedlings: diploid (a), triploid (b), tetraploid (c), aneuploid (d)

Table 3. Ploidy level variation in Jonagold progeny based on karyotypic analysis

Source of plantlet	No. of plantlet	No. of the same ploidy level for plantlets			
		diploid	triploid	tetraploid	aneuploidy
in vitro and plump-seeds	436	179	5	3	249
in vitro and shriveled-seeds	45	12	2	1	30
Common stratification and germination and plump-seeds	192	29	2		161
Common stratification and germination and shriveled-seeds	17	5			12

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Table 4. Ploidy level variation in Jonagold seedlings based on the flow cytometry determination of nuclear DNA contents (± standard deviation)

Estimate ploidy	Mean G1	Relative nuclear DNA content	Ratio
Ck (diploid)	47.2	100	
Diploid	50.9 ± 0.08	107.84 ± 0.08	1.07
Triploid	72.8 ± 0.12	154.24 ± 0.12	1.54
Tetraploid	103.8 ± 0.11	219.92 ± 0.11	2.20
Aneuploid	67.2 ± 0.21	142.37 ± 0.21	1.42

Ck – control check; G1 – G1 phase, interphase begins with G_1 (G stands for gap) phase; during this phase, the cell makes a variety of proteins that are needed for DNA replication

the areas marked C, D and E defined the presence of diploid, triploid and tetraploid nuclei, respectively. The DNA content of diploid nuclei was estimated to be 50.9 ± 0.08, of triploid ones 72.8 ± 0.12 and of tetraploid ones 103.8 ± 0.11. The aneuploid nuclei were identified as those harbouring an intermediate DNA content (67.2 ± 0.21) (Table 4).

Variation in leaf shape and number of stomata among the Jonagold progeny. Variation in leaf shape and in the number of stomata present in the guard cells was characterized for 39 seedlings (13 euploids, 13 diploids and 13 aneuploids). The euploid individuals formed a larger and thicker leaf lamina, which was more intensely green and tended to be rounder than in the diploids (Figure 3a, b); their mean leaf length was 7.12 cm, width 5.04 cm and shape index 1.51 (Table 5). In contrast, the aneuploid seedlings produced small, thin leaves (Figure 3d), which were on average 3 cm shorter and 3 cm narrower than the euploid ones, and had a shape index > 2 (Table 5). The leaves of the diploids were intermediate (Figure 3c). Stomatal density ranged from 19.7 in the polyploids to 38.5 in the aneuploids (Table 6), while the length and width of the guard cells were 26.3 and 32.6 cm (euploids), 14.8 and 20.5 cm (diploids), and 11.0 and 17.0 cm (aneuploids). Chloroplast numbers varied in the same direction as the guard cell size. Stomatal density in the euploid seedlings was relatively low, although their size was larger; the number of chloroplasts harboured by guard cells was greater than that of either diploids or aneuploids (Figure 4).

Table 5. Leaf shape index variation in Jonagold seedlings (± standard deviation)

Ploidy level for plantlets	Leaf length (cm)	Leaf width (cm)	Leaf shape index
Polyploid	7.12 ± 0.21	5.04 ± 0.14	1.51 ± 0.17
Diploid	6.04 ± 0.11	3.21 ± 0.06	1.88 ± 0.08
Aneuploidy	4.27 ± 0.06	2.09 ± 0.08	2.07 ± 0.09

***S*-locus and SSR genotyping**. A sample of seven triploid seedlings was genotyped with respect to the *S*-locus. The parental Jonagold *S*-locus genotype was defined by the amplification of a fragment when the DNA template was primed by pairs S2, S3 and S9,

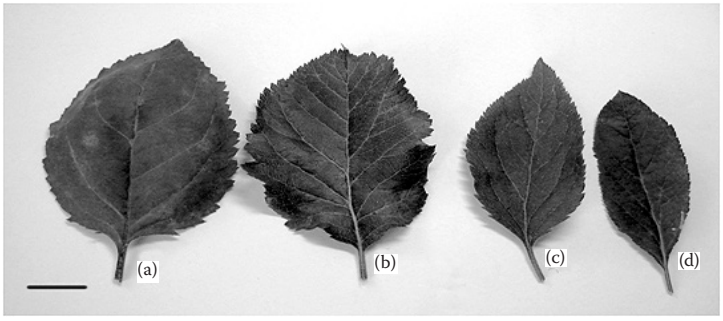

Figure 3. Leaf shape variation in Jonagold seedlings with different ploidy levels: tetraploid (a), triploid (b), diploid (c), aneuploid (d); bar: 1.5 cm

Table 6. Characterization of variation for stomatal density, size and chloroplast density in Jonagold seedlings (± standard deviation)

Ploidy level of plantlets	Density of stomata (No./mm^2)	Guard cell length (µm)	Guard cell width (µm)	No. of chloroplasta/mm^2
Polyploid	19.73 ± 0.10	26.27 ± 0.09	32.65 ± 0.14	22.30 ± 0.11
Diploid	29.50 ± 0.17	14.85 ± 0.20	20.51 ± 0.15	15.35 ± 0.15
Aneuploidy	38.47 ± 0.28	10.97 ± 0.18	16.96 ± 0.14	12.15 ± 0.13

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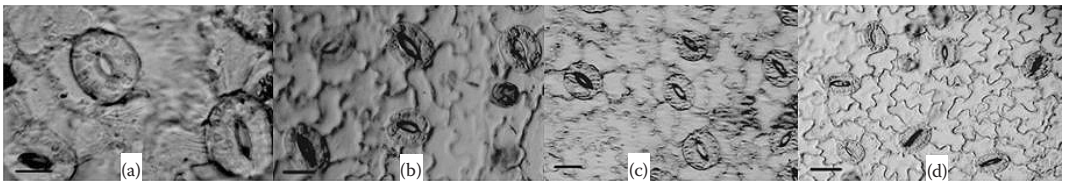

Figure 4. Variation of stomatal size in Jonagold seedlings with different ploidy levels: tetraploid (a), triploid (b), diploid (c), aneuploid (d); bar: 50 μm

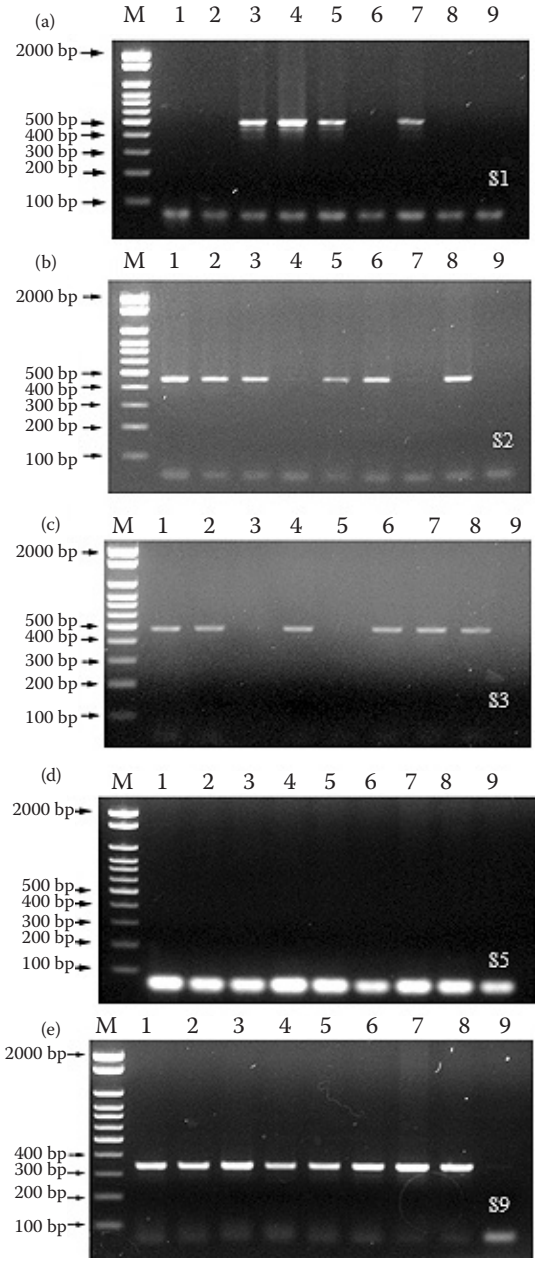

Figure 5. Polymorphism in the *S*-locus based on amplicons using primers for alleles *S1* (a), *S2* (b), *S3* (c), *S5* (d) and *S9* (e); M – DNA weight marker (100 bp ladder); lanes: 1 – Jonagold, 2–8 – triploid seedlings No. 1 through No. 7; 9 – control without DNA

and this genotype was shared by progenies No. 1, 5 and 7 (lanes 2, 4 and 8 in Figure 5). The templates prepared from the other four seedlings (No. 2, 3, 4 and 6), when amplified with primers FTC168 and FTC169 (*S1* allele), each seedling provided a fragment which was not amplified in Jonagold. The S2 profiles of progenies No. 3 and 6 lacked the fragment amplified in Jonagold. Similarly progenies No. 2 and 4 lacked the Jonagold' S3 fragment. All seven seedlings, along with Jonagold, provided a fragment when the template was amplified with primers FTC154 and FTC155 (*S9* allele). When seedlings No. 3, 5 and 7 were subsequently genotyped at the five selected SSR loci, a polymorphism was identified in the CH01F09 locus, but not in any of the other four ones (Figure 6).

DISCUSSION

Polyploidy has played a major role in the evolution of the angiosperms (Soltis *et al*. 2003). It has long been understood that higher ploidy levels can induce an increase to the overall size of the plant as well as to its constituent organs. A relationship has been established in certain species between the ploidy level and both the size of the stomata and the number of chloroplasts harboured by guard cells (Rensing 2014; Soltis *et al*. 2015). Here, triploidy and tetraploidy in apple were shown to be associated with the formation of larger, thicker leaves which are darker green than those formed by diploids; they also

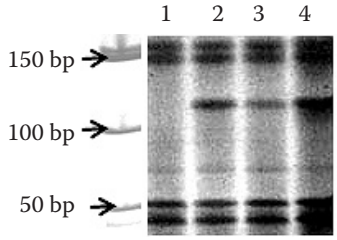

Figure 6. Polymorphism among selected Jonagold seedlings in the SSR locus CH01f09; lanes: 1 – Jonagold; 2 – seedling No. 3; 3 – seedling No. 5; 4 – seedling No. 7

https://doi.org/10.17221/201/2016-CJGPB

develop a rounder leaf, a smaller leaf shape index, smaller stomata and larger guard cells. Aneuploid plants, however, tended to be weak.

Both triploid and tetraploid offspring can be generated from a triploid mother plant by pollinating with a diploid donor. The number of viable seeds produced by these crosses tends to be limited, with most of the progeny being aneuploid. In a survey of 884 progenies of a triploid mother plant harvested in an orchard containing many diploid trees, only eight proved to be tetraploid (Laubscher & Hurter 1960); similarly, Einset (1952) showed that the open-pollinated progeny of triploids included a very low (0.6%) proportion of haploids and only a few euploids (1.8% diploids, 1.8% triploids and 3.0% tetraploids), with an overwhelming proportion (92.8%) of progeny being aneuploid. Later research by the same author suggested that not all triploids respond in this way, as some are able to produce a higher proportion of polyploids (Einset 1952). Here, the proportion of polyploid (triploid and tetraploid) progeny obtained from the triploid parent was just 1.9%, but the proportion of diploids was as high as 32.6%.

The chromosome number of the offspring of a plant characterized by non-diploid-like meiosis is unpredictable. When one parent of an apple cross is triploid, the female gamete chromosome complement can be highly variable, including the three possible complete complements of n = 17, 34 or 51. The fusion of euploid gametes with a haploid gamete (n = 17) will produce a diploid, triploid and tetraploid hybrid. Other possible routes to polyploidy include the formation of unreduced pollen ($n = 2x$ = 34) or apomixis in the mother plant. The former route has been observed in a range of fruit species (Sanford 1983; Passvetaeva 1985; Jackson and Chen 2010; Jiao *et al*. 2011), while apomixis is known to occur in 300 plant species at least (Levin 2002). Among the seven triploid progenies of Jonagold, three shared the same *S*-locus genotype as Jonagold and existed different in codomain. The implication is that for these progenies, the maternal stigma supported the growth of pollen carrying some of the same *S*-locus alleles. The genotypic analysis of the *S*-locus and the selected SSR loci suggested that the triploid progeny must have originated from the fusion of an $n = 2x$ = 34 female gamete with an $n = x$ = 17 male gamete, or *vice versa*, rather than via apomixis.

Acknowledgments. The authors thank the Changli Institute of Pomology (Hebei Academy of Agricultural and Forestry Sciences of China) for providing access to flow cytometry. We acknowledge the financial support of the Fruit Germplasm Resources Collection and New Germplasm Innovation (Lu No. 2014-96), National Science and Technology Plans Program (2013BAD02B01) and China Agriculture Research System (CARS-27).

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Received for publication December 27, 2016
Accepted after corrections February 9, 2018
Published online April 23, 2018